REMARKS

Prior to the present amendment, claims 1 to 8, 45 to 53, 55 and 57 to 67 were pending. Claims 1 to 3 have been canceled, and new claims 96 to 148 have been added herein. Thus, claims 4 to 8, 45 to 53, 55, 57 to 67 and 96 to 148 are presently under examination.

Regarding the amendment to the specification

The specification has been amended at page 40 to correct a typographical error. In particular, the sentence at page 40, lines 4-11, has been amended to indicate that a scissile bond cleaved by BoNT/A can be, for example, "Gln-Arg" rather than "Gln-Ala." The amendment to page 40 is supported throughout the specification as filed, for example, at page 39, lines 16-20, which indicates that BoNT/A substrates can include at least six consecutive residues of SNAP-25 including "Gln-Arg," and at page 31, Table 1, line 8, which indicates that the SNAP-25 bond cleaved by BoNT/A is "Gln-Arg." In sum, the amendment to the specification is supported by the application as originally filed and does not add new matter. Accordingly, the Examiner is respectfully requested to enter the amendment to the specification.

Regarding the claim amendments and new claims

Claim 4 has been amended herein to recite specific lengths of the claimed botulinum toxin serotype A (BoNT/A) substrate. The amendment to claim 4 is supported throughout the specification, for example, at page 40, lines 13-15, and page 115, lines 3-15, which discloses specific lengths of BoNT/A substrate. New claims 96 to 101 depend from claim 4 and separately recite specific lengths of BoNT/A substrate. Furthermore, in view of the cancellation of claims 1 to 3, claims 45 to 50, 52, 53, 55 and 57 to 60 have been amended to depend solely on claim 4. The amendments to claims 4, 45 to 50, 52, 53, 55 and 57 to 60 are supported by the application as filed and do not add new matter.

Claim 55 further has been amended to recite the full name for the chemical represented by the well-known abbreviation "DABCYL," 4-(4-dimethylaminophenylazo)benzoic acid. Similarly, claim 58 has been amended to recite the full name for the chemical represented by the well-known abbreviation "EDANS," 5-[(2-aminoethyl)amino]-naphthalene-1-sulfonic acid. The amendments to claims 55 and 58 are supported throughout the specification, for example, at page

83, lines 7-8, and page 116, lines 21-24, which incorporates by reference Wang et al., Anal. Biochem. 210:351-359 (1993), attached hereto as Exhibit A for the Examiner' convenience. Wang et al. explicitly recite the well-known definitions for the abbreviations DABCYL and EDANS at page 351, footnote 2. Thus, the amendments to claims 55 and 58 are supported by the specification as filed and do not add new matter.

New claims 102 to 125 have been added herein. New claim 102 is supported in the application as filed, for example, by original claim 6 and in the specification, for example, at page 39, lines 7-23, which indicates that a BoNT/A substrate of the invention can have, for example, at least six consecutive residues of human SNAP-25, where the six consecutive residues include Gln_{197}-Arg_{198}, or a peptidomimetic thereof, and at page 17, line 18, which indicates that human SNAP-25 has the amino acid sequence SEQ ID NO: 2. New claim 103 is supported by original claim 7, and new claim 104 is supported by original claim 8 and in the specification, for example, at page 40, lines 26-29, which discloses the BoNT/A recognition sequence Ser-Asn-Lys-Thr-Arg-Ile-Asp-Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys-Met-Leu (SEQ ID NO: 30). New claims 105 to 116 are supported by original claims 45 to 53, 55, 57 and 58, respectively, and new claims 118 to 125 are supported by original claims 60 to 67, respectively. New claim 117, directed to a BoNT/A substrate of the invention in which the acceptor is a non-fluorescent acceptor, is supported throughout the specification as filed, for example, at page 20, lines 16-29, which indicates that acceptors useful in the substrates of the invention include non-fluorescent acceptors.

New claims 126 to 148 have been added herein. These new claims are supported by the specification and claims as originally filed and do not add new matter. In particular, new claims 126 to 129 are supported, for example, by original claim 4 and at page 86, line 12, to page 87, line 8, which describes the use of genetically encoded donor fluorophore or acceptor. New claims 130 to 148 are supported, for example, by original claims 5 to 8, 45 to 51, and 60 to 67.

As set forth above, each of the amendments and new claims is supported by the application as originally filed and does not add new matter. Applicants therefore respectfully request that the Examiner enter the amendments and new claims.

Regarding the rejection under 35 U.S.C. § 112, second paragraph

The rejection of claims 55 and 58 under 35 U.S.C. § 112, second paragraph, as allegedly vague and indefinite is respectfully traversed. Applicants submit that the term "DABCYL" is a well known abbreviation for 4-(4-dimethylaminophenylazo)benzoic acid. See, for example, Wang et al., Anal. Biochem. 210:351-359 (1993), especially page 351, footnote 2. This reference, attached as Exhibit A, is incorporated into the specification at page 83, lines 7-8 (see, also, page 116, lines 21-24). Although Applicants maintain that the term "DABCYL" is clear as written, claim 55 has been amended in order to further prosecution to more clearly indicate that the recited donor fluorophore is "4-(4-dimethylaminophenylazo)benzoic acid."

Similarly, Applicants respectfully submit that the term "EDANS" is an abbreviation well known in the art for the chemical 5-[(2-aminoethyl)amino]-naphthalene-1-sulfonic acid as set forth, for example, in footnote 2 of Wang et al., attached hereto as Exhibit A. While Applicants maintain that the term "EDANS" is clear as written, claim 58 has been amended in order to further prosecution of the subject application to more clearly indicate that the recited acceptor is "5-[(2-aminoethyl)amino]-naphthalene-1-sulfonic acid." In view of the above remarks and amendments, the Examiner is respectfully requested to reconsider and remove the rejection of claims 55 and 58 as allegedly vague and indefinite under the second paragraph of 35 U.S.C. § 112.

Regarding objections to the claims

The objection to claims 57 and 59 for depending on canceled claim 54 is respectfully traversed. Claims 57 and 59 have been amended herein to depend from claims 1 and 53 rather than claims 1, 53 and 54. In view of the amendment of the claim dependency of claims 57 and 59, Applicants respectfully request that the Examiner reconsider and withdraw the claim objections.

Regarding the rejection under § 102(e)

The rejection of claims 1 to 8, 45 to 54 and 60 to 67 under 35 U.S.C. § 102(e) as allegedly anticipated by Schmidt et al. (U.S. Patent No. 6,762,280) is respectfully traversed. In

view of the cancellation of claims 1 to 3 and 54, this rejection will be addressed as it pertains to pending claims 4 to 8, 45 to 53 and 60 to 67.

The cited patent by Schmidt et al. allegedly describes substrates for clostridial toxins, including modified peptides or proteins useful as FRET substrates (abstract and column 4). In particular, the cited patent by Schmidt et al. allegedly reports FRET clostridial toxin substrates in which a fluorescent group and a molecule that quenches fluorescence are positioned on either side of a cleavage site (columns 5 and 7) and further allegedly describes the human SNAP-25 sequence and the BoNT/A recognition sequences of SEQ ID NOS: 1 and 2.

Applicants respectfully note that U.S. Patent No. 6,762,280 (the '280 patent) is cited as a §102(e) reference based on the filing date of the priority provisional application Serial No. 60/235,050, filed on September 25, 2000. The actual filing date of the application which matured into the '280 patent, September 25, 2001, does not predate the subject application, which has a filing date of August 28, 2001. Thus, Applicants respectfully remind the Examiner that the '280 patent is proper §102(e) prior art only for the subject matter disclosed in the priority provisional application Serial No. 60/235,050, filed on September 25, 2000. For the Examiner's convenience, a copy of priority provisional application serial No. 60/235,050 is attached as Exhibit B.

The BoNT/A substrates of claims 4 to 8, 45 to 53 and 60 to 67 are novel over U.S. Patent No. 6,762,280. Claim 4, as amended, is directed to a botulinum toxin serotype A (BoNT/A) substrate containing a donor fluorophore; an acceptor having an absorbance spectrum overlapping the emission spectrum of the donor fluorophore; and a BoNT/A recognition sequence containing a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and the acceptor, where the substrate has a length of amino acids selected from the group consisting of 19 amino acids, 20 amino acids, 21 amino acids, 22 amino acids, 69 amino acids and 72 amino acids. In contrast, the priority provisional application Serial No. 60/235,050, filed on September 25, 2000, at best describes a single short BoNT/A synthetic peptide substrate in which a fluorescent group and a molecule that quenches fluorescence are positioned on either side of a cleavage site. The sequence of the

synthetic BoNT/A peptide substrate is set forth at page 1 of the priority provisional application Serial No. 60/235,050, as follows:

N(alpha)-acetyl-S N R T R I D X A N **Q R** A Z R M L (amide), in which "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and "Z" is S-(fluoresceinyl)-cysteine.

Applicants assert that the priority provisional application Serial No. 60/235,050 does not describe the claimed BoNT/A substrate, which has a length of amino acids selected from the group consisting of 19 amino acids, 20 amino acids, 21 amino acids, 22 amino acids, 69 amino acids and 72 amino acids. Therefore, the cited 102(e) reference cannot anticipate the invention.

Accordingly, Applicants respectfully request that the Examiner reconsider and remove the rejection of claims 1 to 8, 45 to 54 and 60 to 67 under 35 U.S.C. § 102(e) as allegedly anticipated by Schmidt et al., U.S. Patent No. 6,762,280.

Regarding the rejection under § 103(a)

The rejection of claims 55 and 57 to 59 under 35 U.S.C. § 103(a) as allegedly obvious over Schmidt et al. (U.S. Patent No. 6,762,280) in view of Holskin et al., Anal. Biochem. 227:148-155 (1995), is respectfully traversed. Claims 55 and 57 to 59 are directed to BoNT/A substrates in which the recited donor fluorophore is DABCYL (claim 55) or in which the recited acceptor is tetramethylrhodamine (claim 57), EDANS (claim 58), or a non-fluorescent acceptor (claim 59).

As stated above, the cited patent by Schmidt et al. allegedly describes a BoNT/A FRET substrate in which a fluorescent group and a molecule that quenches fluorescence are present on either side of a cleavage site (peptide 1, page 1). While the Office Action acknowledges that Schmidt et al. do not teach EDANS and DABCYL, Holskin et al. is cited as allegedly describing the DABCYL-EDANS donor-acceptor pair and the use of this donor-acceptor pair in fluorescence-based assays for proteases such as the HIV protease and renin. The Office Action concludes that the use of a SNAP-25 recognition sequence in a substrate with the DABCYL-EDANS FRET pair would have been obvious to a person of ordinary skill in the art at the time the invention was made.

Applicants assert that the BoNT/A substrates of claims 55, 57, 58 and 59 are unobvious over U.S. Patent No. 6,762,280 in view of Holskin et al. Specifically, each of claims 55, 57, 58, and 59 depends from claim 4 and, therefore, is directed to a BoNT/A substrate having a length of amino acids selected from the group consisting of 19 amino acids, 20 amino acids, 21 amino acids, 22 amino acids, 69 amino acids and 72 amino acids. In contrast, neither of the cited references teaches nor suggests a BoNT/A substrate having a length of amino acids selected from the group consisting of 19 amino acids, 20 amino acids, 21 amino acids, 22 amino acids, 69 amino acids and 72 amino acids. At best, the secondary reference by Holskin et al. appears to describe the donor-acceptor pair, DABCYL-EDANS and its use as a substrate for other proteases, but does not teach or suggest the claimed BoNT/A substrates. Furthermore, as discussed above, the priority provisional application Serial No. 60/235,050, filed on September 25, 2000, at best describes a relatively poor synthetic BoNT/A FRET substrate. Absent such a teaching or suggestion, the BoNT/A substrates of claims 55, 57, 58 and 59 are unobvious over U.S. Patent No. 6,762,280 in view of Holskin et al.

In view of the above remarks, Applicants respectfully request that the Examiner reconsider and remove the rejection of claims 55, 57, 58 and 59 under 35 U.S.C. § 103(a) as allegedly unpatentable over U.S. Patent No. 6,762,280 in view of Holskin et al.

Regarding new claims 102 to 125

New claims 102 to 125 are directed to a BoNT/A substrate that includes (a) a donor fluorophore; (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of the donor fluorophore; and (c) a BoNT/A recognition sequence containing a cleavage site and at least six consecutive residues of human SNAP-25 (SEQ ID NO: 2), including the sequence Gln197-Arg198 or a peptidomimetic thereof, where the cleavage site intervenes between the donor fluorophore and the acceptor and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and acceptor. Thus, the BoNT/A recognition sequence included in the BoNT/A substrates of new claims 102 to 125 has at least six residues of the wild type human SNAP-25 sequence, with these six residues including, in part, the Gln_{197}-Arg_{198} cleavage site.

In contrast to the BoNT/A substrates of new claims 102 to 125, Schmidt et al. do not describe a BoNT/A substrate having at least six consecutive residues of human SNAP-25 (SEQ ID NO: 2) including the Gln_{197}-Arg_{198} cleavage site. As can be readily appreciated by review of Table 1 below, the Schmidt et al. peptide differs from the wild type human SNAP-25 (SEQ ID NO: 2) sequence in the region of six consecutive residues including the Gln_{197}-Arg_{198} cleavage site. In particular, in the BoNT/A peptide of Schmidt et al., a region of six consecutive residues including the Gln_{197}-Arg_{198} cleavage site will necessarily contain either residue "X," a modified lysine residue substituted for the wild type human SNAP-25 residue glutamate, or the residue "Z," a modified cysteine residue substituted for the wild type human SNAP-25 residue threonine. Furthermore, as discussed above, Holskin et al. does not teach or suggest the claimed BoNT/A substrates containing at least six consecutive residues of human SNAP-25 (SEQ ID NO:2) including the Gln_{197}-Arg_{198} cleavage site. In the absence of a description of a BoNT/A substrate which contains at least six consecutive residues of human SNAP-25 (SEQ ID NO: 2) including the Gln_{197}-Arg_{198} cleavage site, the invention of new claims 102 to 125 is unobvious over Schmidt et al., alone or in combination with Holskin et al.

Table 1

Comparison of the wild type human SNAP-25 sequence surrounding the BoNT/A cleavage site with the synthetic BoNT/A peptide 1 of Schmidt et al.

Residue position	193	194	195	196	**197**	**198**	199	200	201	202
human SNAP-25 (SEQ ID NO: 2)	Asp	Glu	Ala	Asn	**Gln**	**Arg***	Ala	Thr	Lys	Met
Schmidt et al. peptide 1	Asp	X	Ala	Asn	**Gln**	**Arg***	Ala	Z	Arg	Met

where X = N(epsilon)-(2,4-dinitrophenyl)-lysine and Z = S-(fluoresceinyl)-cysteine
* cleavage site shown in bold

Regarding New Claims 126 to 148

New claims 126 to 148 are directed to botulinum toxin serotype A (BoNT/A) substrates containing a donor fluorophore; an acceptor having an absorbance spectrum overlapping the emission spectrum of said donor fluorophore; and a BoNT/A recognition sequence containing a cleavage site, where the cleavage site intervenes between the donor fluorophore and the acceptor

and where, under the appropriate conditions, resonance energy transfer is exhibited between the donor fluorophore and the acceptor, where the donor fluorophore or the acceptor is genetically encoded.

In contrast to the substrates of new claims 126 to 148, Schmidt et al. does not teach or suggest the use of a genetically encoded fluorophore or acceptor. At best, Schmidt et al. describes the chemical compounds "fluorescein and dinitrophenyllysine" as examples of "signal and quench moeities [sic]" (Exhibit B, page 2, first paragraph). Moreover, Holskin et al. does not teach or suggest the use of a genetically encoded fluorophore or acceptor. Accordingly, the invention of new claims 126 to 148 is unobvious over Schmidt et al., alone or in combination with Holskin et al.

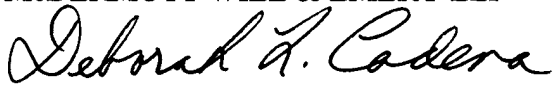
CONCLUSION

The Examiner is respectfully requested to consider the above remarks. The Examiner is invited to call the undersigned agent or Cathryn Campbell if there are any questions.

To the extent necessary, a petition for an extension of time under 37 C.F.R. 1.136 is hereby made. Please charge any shortage in fees due in connection with the filing of this paper, including extension of time fees, to Deposit Account 502624 and please credit any excess fees to such deposit account.

Respectfully submitted,

McDERMOTT WILL & EMERY LLP



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ANALYTICAL BIOCHEMISTRY 210, 351–359 (1993)

A Continuous Fluorescence Assay of Renin Activity

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A sensitive fluorescence assay that employs a new fluorogenic peptide substrate has been developed to continuously measure the proteolytic activity of human renin. The substrate, DABCYL-gaba–Ile–His–Pro–Phe–His–Leu–Val–Ile–His–Thr–EDANS, has been designed to incorporate the renin cleavage site that occurs in the N-terminal peptide of human angiotensinogen. The assay relies upon resonance energy transfer-mediated, intramolecular fluorescence quenching that occurs in the intact peptide substrate. Efficient fluorescence quenching occurs as a result of favorable energetic overlap of the EDANS excited state and the DABCYL absorption, and the relatively long excited state lifetime of the EDANS fluorophore. Cleavage of the substrate by renin liberates the peptidyl-EDANS fragment from proximity with the DABCYL acceptor, restoring the higher, unattenuated fluorescence of the EDANS moiety. This leads to a time-dependent increase in fluorescence intensity, directly related to the extent of substrate consumed by renin cleavage. The kinetics of renin-catalyzed hydrolysis of this substrate have been shown to be consistent with a simple substrate inhibition model with a substrate $K_m \cong 1.5$ μM at physiological pH; Cleavage of the substrate occurs specifically at the Leu–Val bond and corresponds to the renin cleavage site of angiotensinogen, as reported earlier. In this report, we describe in detail the synthesis of the fluorogenic renin substrate and its application in assays of renin activity. Assay sensitivity has been evaluated by a series of enzyme dilution experiments using the continuous assay format, showing that the assay can detect renin as low as 30 ng/ml after a incubation of only 3–5 min. It was estimated that with extended incubation time (2–3 h) the assay can detect renin at 0.5 ng/ml concentration level. An automated, high throughput fluorometric renin assay has been developed for a 96-well microtiter-plate fluorescence reader, which is useful for studies of enzyme inhibitors and enzyme stability. © 1993 Academic Press, Inc.

Renin (EC 3.4.99.19) is an aspartic protease that represents an attractive target for therapeutic intervention in hypertension (1). Renin is an important enzyme in blood pressure regulation and electrolyte homeostasis through the well-known renin–angiotensin system (see 2–4 for reviews). Upon release into the bloodstream, renin specifically hydrolyzes the glycoprotein angiotensinogen to form angiotensin I, which in turn is hydrolyzed by angiotensin converting enzyme to generate angiotensin II. This octapeptide is a potent vasoconstrictor and a promoter of aldosterone release and sodium retention. High renin activity levels also have been associated with heart disease (5–7), and recent evidence from a clinical study by Laragh and co-workers (8) strongly suggests that renin activity is a distinct and independent risk factor for heart attack.

Renin activity is typically measured in a time point assay by radioimmunoassay of angiotensin I, using a rabbit polyclonal antibody (9–11). A kinetic profile of renin activity can be obtained by making sequential RIA[2] measurements, though this is impractical for assaying large numbers of renin reactions, and impedes detailed kinetic analysis of renin. Several other types of assays for renin activity also have been published. A fluorescence assay based on a C-terminal amide of β-naphthylamine or 7-amino-4-methylcoumarin has been described (12,13), in which renin carries out the initial endoproteolytic event to generate a carboxy terminal fragment, which in turn is degraded by the action of an aminopeptidase enzyme to the free fluorescent label. This coupled fluorescence assay is potentially simpler and easier to use than the RIA, but it is not a

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[2] Abbreviations used: RIA, radioimmunoassay; HIV, human immunodeficiency virus; EDANS, 5-[(2-aminoethyl)amino]-naphthalene-1-sulfonic acid; DABCYL, 4-(4-dimethylaminophenylazo)benzoic acid; DMF, dimethylformamide; DCC, dicyclohexylcarbodiimide; NHS, *N*-hydroxysuccinimide; DCU, *N,N'*-dicyclohexylurea; gaba, γ-aminobutyric acid; TFA, trifluoroacetic acid; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; TPCK, L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone; Mops, 4-morpholinepropane sulfonic acid; FU, fluorescence units.

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continuous assay and it requires multiple time point measurements to assess enzyme kinetic parameters. An HPLC-based assay employing a synthetic tetradecapeptide substrate suffered from the same drawback (14).

Recently, we described a rapid and continuous fluorogenic assay for the dimeric aspartic protease associated with human immunodeficiency virus (HIV) (15–17). This assay employed an internally quenched fluorogenic substrate consisting of a fluorophore, 5-[(2-aminoethyl)amino]-naphthalene-1-sulfonic acid (EDANS), and a quenching chromophore, 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL), linked by an octapeptide that represents one of several native cleavage sites within the HIV *gag* polyprotein. Cleavage of the weakly fluorescent substrate liberates a highly fluorescent tetrapeptide–fluorophore fragment, and the increase in fluorescence can be related directly to the extent of substrate cleavage. This approach is quite versatile and can be adapted to assay virtually any peptidase enzyme, or other hydrolase enzyme, since fluorophore and quencher can be attached to a variety of connecting substrate components.

In this paper, we describe the synthesis of an internally quenched fluorogenic peptide substrate based on the renin cleavage site of angiotensinogen (Fig. 1), and we detail applications of this new substrate for renin purification, enzymology, renin inhibitor evaluation and automated inhibitor screening.

MATERIALS AND METHODS

Synthesis of Fluorogenic Substrate (1)

Synthesis of DABCYL–gaba–N-hydroxysuccinimide. 4-(4-dimethylaminophenylazo)-benzoic acid sodium salt (Sigma, 5.0 g, 17.1 mmol) was dissolved in 1.0 liter of boiling water and 0.5 liter of a pH 4.0 (0.1 M NaOAc) buffer was added. After cooling, the precipitate was collected by filtration and dried to give 4-(4-dimethylaminophenyl-azo)-benzoic acid (4.6 g, 100% yield). This material (DABCYL–OH, 4.0 g, 14.8 mmol) was dissolved in 300 ml of dimethylformamide (DMF), and dicyclohexylcarbodiimide (DCC, Aldrich, 3.7 g, 17.8 mmol) and *N*-hydroxysuccinimide (NHS, Aldrich, 2.05 g, 17.8 mmol) were added to the solution. Reaction progress was monitored by thin-layer chromatography (TLC), and upon completion, the DMF was removed under reduced pressure. The residue was taken up in 400 ml of dichloromethane and filtered to remove the insoluble by-product, *N,N'*-dicyclohexylurea (DCU). The dichloromethane was evaporated to provide the active ester DABCYL–NHS (4.6 g, 80% yield). This material (4.0 g, 10.9 mmol) was dissolved in DMF (300 ml), followed by the addition of γ-aminobutyric acid (gaba, Aldrich, 1.23 g, 12 mmol) and triethylamine (Aldrich, 2.0 ml, excess). After stirring at room temperature overnight, the DMF solvent was evaporated, and the residue was recrystallized from methanol–dichloromethane mixture to provide pure DABCYL–gaba–OH.

DABCYL–gaba–OH (2.0 g, 5.6 mmol.) was dissolved in dry DMF (300 ml) and treated with 0.8 g NHS (1.2 eq) and 1.4 g DCC (1.2 eq). The reaction was complete after stirring overnight at room temperature. The mixture was filtered and the filtrate was concentrated. The residual red solid was taken up in 300 ml of dichloromethane and filtered in order to remove remaining DCU. The dichloromethane solution applied directly to a silica gel chromatography column, and eluted with EtOAc to provide 1.58 g DABCYL–gaba–NHS, which was homogeneous by TLC. MS (FAB+), $(M + 1) = 452$.

Synthesis of Ile–His–Pro–Phe–His–Leu–Val–Ile–His–Thr. This decapeptide was synthesized on an Applied Biosystems Inc. (Foster City, CA) Model 430A peptide synthesizer using standard Fmoc chemistry. Fmoc amino acids with appropriate protecting groups were purchased from Applied Biosystems. Cleavage of the peptide from the resin and deprotection was achieved by reaction with trifluoroacetic acid containing 7% v/v of a scavenger mixture (ethanedithiol: anisole:ethyl methyl sulfide, 1:3:3) for 2 h at room temperature. Addition of diethyl ether to the residue precipitated the peptide, which was collected and dried. The peptide was carried forward without additional purification. MS (FAB+). $(M + 1) = 1213$.

Synthesis of DABCYL–gaba–Ile–His–Pro–Phe–His–Leu–Val–Ile–His–Thr. To a solution of the decapeptide Ile–His–Pro–Phe–His–Leu–Val–Ile–His–Thr (49.0 mg, 0.046 mmol) in 10 ml of dry DMF was added triethylamine (0.2 ml, excess) and 30 mg (0.085 mmol) of DABCYL–gaba–NHS. After stirring at room temperature overnight, the mixture was concentrated to dryness. The red residue was washed with dichloromethane several times until the washing was colorless, in order to remove excess unreacted starting material DABCYL–gaba–NHS from the functionalized peptide. Filtration of the final dichloromethane suspension provided 60 mg of a red solid as the desired product. MS (FAB+). $(M + 1) = 1548$, $(M + Na) = 1570$.

Synthesis of DABCYL–gaba–Ile–His–Pro–Phe–His–Leu–Val–Ile–His–Thr–EDANS. DABCYL–gaba–Ile–His–Pro–Phe–His–Leu–Val–Ile–His–Thr (50.0 mg, 0.039 mmol) was dissolved in dry DMF (20 ml). To this solution was added EDANS (Sigma, 17 mg, 0.058 mmol), NHS (10 mg, excess), and 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide hydrochloride (Sigma, 25 mg, excess). After stirring at room temperature for 5 h, reverse-phase HPLC analysis indicated complete conversion of the starting DABCYL–gaba–Ile–His–Pro–Phe–His–Leu–Val–Ile–His–Thr to a new component.

The mixture was filtered and purified directly by HPLC using a Rainin Dynamax C-8 reverse-phase column (21 × 250 mm) with a flow rate of 12 ml/min, eluting with 0.1% aqueous TFA for 10 min, followed by a linear gradient to 60% acetonitrile over a period of 50 min. When a dual-channel uv–vis detector was used, a ratio of 2.5:1 for absorptions at 490 nm (λ_{max} of the DABCYL moiety) and 260 nm (λ_{max} of the EDANS moiety) served as a convenient analytical parameter to identify the eluting peak containing the desired product. Fractions that contained the desired product were combined and concentrated to give a crystalline red solid (49.0 mg). MS (FAB+): $(M + 1) = 1819$, $(M + Na) = 1840$.

Continuous Fluorogenic Assay of Renin Activity

Recombinant prorenin was obtained from a Chinese hamster ovary cell expression system and purified to homogeneity (19). Activation of prorenin to renin followed the procedure of Egan *et al.* (21). A sample of prorenin (60 μl) was incubated with 30 μl of a 10 wt% BSA solution and 300 μl of a 10 μg/ml TPCK-treated trypsin (Worthington Biochemicals) solution in a pH 6.5 buffer containing 50 mM Mops, 200 mM NaCl, and 1 mM EDTA. After 30 min at room temperature, the activation was terminated by addition of 30 μl of 2 mg/ml soybean trypsin inhibitor (Sigma Chemicals). Renin concentration was determined from the concentration of pure prorenin, as measured spectroscopically using a converting factor of 1.0 mg/ml per absorbance unit at 280 nm (T. F. Holzman *et al.*, unpublished data).

Fluorogenic enzyme activity assays were carried out on a Shimadzu RF-5000U spectrofluorometer equipped with a thermostated cell holder maintained at 37°C using a 3 × 3-mm square microcuvette. The following instrumental parameters were used: excitation wavelength, 340 nm; emission wavelength, 490 nm; excitation bandwidth, 1.5 nm; and emission bandwidth 1.5 or 10 nm. Typically, 80.0 μl of a 0.05 M Tris buffer, pH 8.0, containing 0.1 M NaCl and 1.0 mM EDTA was mixed with 10.0 μl of a DMSO stock solution of the fluorogenic substrate and incubated for several minutes to reach a thermal equilibrium. The reaction was then initiated by addition of 10 μl of a renin stock solution and the time dependent increase of fluorescence intensity was monitored for 3–5 min. The initial reaction velocity, expressed in arbitrary fluorescence units per minute (FU/min), was obtained by least-squares analysis of the initial phase of the reaction.

Fixed-Time Assay Using a Microtiter-Plate Reader

Fixed-time assays were performed on a Perkin–Elmer LS-50 luminescence spectrophotometer with a 96-well microtiter plate attachment at ambient temperature. A 340-nm interference filter was used for excitation and a 490-nm cutoff filter used for emission. The excitation slit was set at 10 nm and the emission slit was set at 15 nm. A total sample volume of 200 μl/well was used. Typically, 160 μl of a 0.05 M Tris buffer, pH 8.0, containing 0.1 M NaCl and 1.0 mM EDTA was mixed with 20 μl of a substrate stock solution in DMSO in each well. Mixing was achieved by repeated pipetting, taking special care to avoid bubble formation. A sample of renin (20 μl) was added to each well with mixing, and the plate was loaded into the instrument. The fluorescence intensity was measured for each well at fixed time points within the linear range of the reaction, and the recorded data were analyzed using the software package EnzFitter (Elsevier Biosoft) to give initial reaction velocities.

RESULTS AND DISCUSSION

The synthesis of the fluorogenic renin substrate 1 proceeded via the scheme outlined in Fig. 1 and was analogous to our previously described synthesis of the fluorogenic substrate for HIV-1 protease (17). The synthesis involved functionalization of a decapeptide containing the P_6–P_3' sequence flanking the cleavage site of human angiotensinogen by renin. The N-terminus of this peptide was labeled with DABCYL–gaba via its *N*-hydroxysuccinimide active ester (DABCYL–gaba–NHS). The C-terminal carboxylic group of this N-terminus labeled peptide was coupled to sodium EDANS via carbodiimide mediated coupling. Purification of the desired substrate was achieved by high-performance liquid chromatography (HPLC).

In a preliminary communication, we reported the utility of this fluorogenic substrate for assaying renin activity (18). HPLC studies and amino acid sequencing results established that fluorogenic substrate cleavage by renin occurred specificallv at the Leu–Val linkage, as expected (20, and references cited therein). The fluorogenic substrate exhibited a maximum velocity at pH 8.0 and the kinetics of the renin catalyzed hydrolysis of this substrate was best described by a substrate inhibition model (20).

Four representative continuous hydrolysis assay traces are presented in Fig. 2. In these experiments, continuous assays were carried out at pH 8.0 as described under Materials and Methods using a final substrate concentration of 20 μM, and renin concentrations of 2.37 μg/ml (A and C), 0.07 μg/ml (B), and 0.03 μg/ml (D). The assay data shown in Figs. 2B and 2D illustrate the dependence of detection limit of the fluorogenic assay on factors such as excitation or emission slit width and incubation time. Although an excitation bandwidth of 1.5 nm was used for all four assay traces shown, the emission bandwidth was 1.5 nm for assay shown in Figs. 2A and 2B, and 10 nm for the assays shown in Figs. 2C

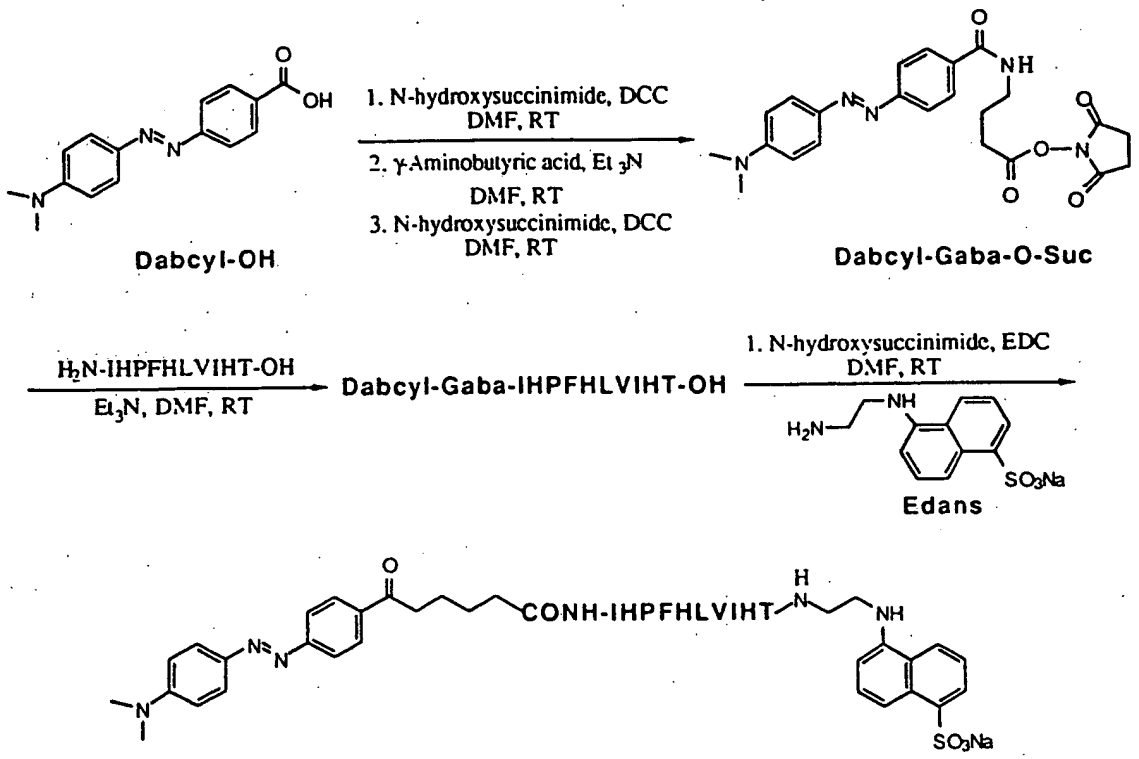

FIG. 1. Structure and synthesis of fluorogenic substrate of renin.

and 2D. The sensitivity of any fluorogenic assay utilizing intramolecular quenched fluorogenic substrates must be dependent primarily on the level of residual fluorescence of the substrate, determined by the quenching efficiency, and the quantum yield of the fluorophore. Comparison of Figs. 2A and 2B with Figs. 2C and 2D illustrates that with a given substrate the sensitivity of the fluorogenic assay (detection limit) is dependent also on the instrument settings, particularly excitation and emission bandwidth, which have a direct impact on the instrument sensitivity. The data shown in Figs. 2B and 2D indicate that the assay, with a incubation time of as short as 3 min, can readily detect the activity of renin at a concentration as low as 70 or 30 ng/ml of renin with the emission slit width set at 1.5 or 10 nm, respectively. Thus, the sensitivity of the fluorogenic assay can be augmented by increasing either the excitation or emission bandwidth. However, the tradeoff of increased bandwidth is higher background fluorescence. In assays involving samples containing fluorescent impurities, overall assay sensitivity may be limited by such sample matrix interference rather than instrument detection capability.

In order to evaluate the sensitivity of the fluorogenic assay, the dependence of the initial velocities on renin concentration was determined. These results are shown in Fig. 3. In these experiments, the initial velocities were measured using the continuous assay protocol, and the values obtained have been expressed in FU/min. The final substrate concentration for these determinations was 20 μM, with renin concentrations established by dilutions of an activated renin stock solution. Figure 3A presents experimental results obtained when both the excitation and the emission bandwidth were set at 1.5 nm, while Fig. 3B presents the results obtained with the excitation bandwidth at 1.5 nm and the emission bandwidth at 10 nm. These results show a linear relationship for both experiments, with renin concentrations up to 1.18 μg/ml. At enzyme concentrations higher than this (e.g., 2.37 μg/ml), significant deviation from linearity was noted.

In order to convert the initial enzyme velocities expressed in fluorescence intensity units to concentration units, calibration experiments were carried out. Ideally, this calibration should be done using Val–Ile–His–Thr–EDANS, the fluorescent product from hydrolysis of the fluorogenic substrate by renin, as the standard. However, for the sake of experimental convenience the free fluorophore EDANS was chosen. It has been shown previously that the fluorescence quantum yield of EDANS was reduced after EDANS was conjugated to a tetrapeptide (15). The ratio of the fluorescence intensity of an equimolar solution of free EDANS to that of the cleavage product Val–Ile–His–Thr–EDANS is referred

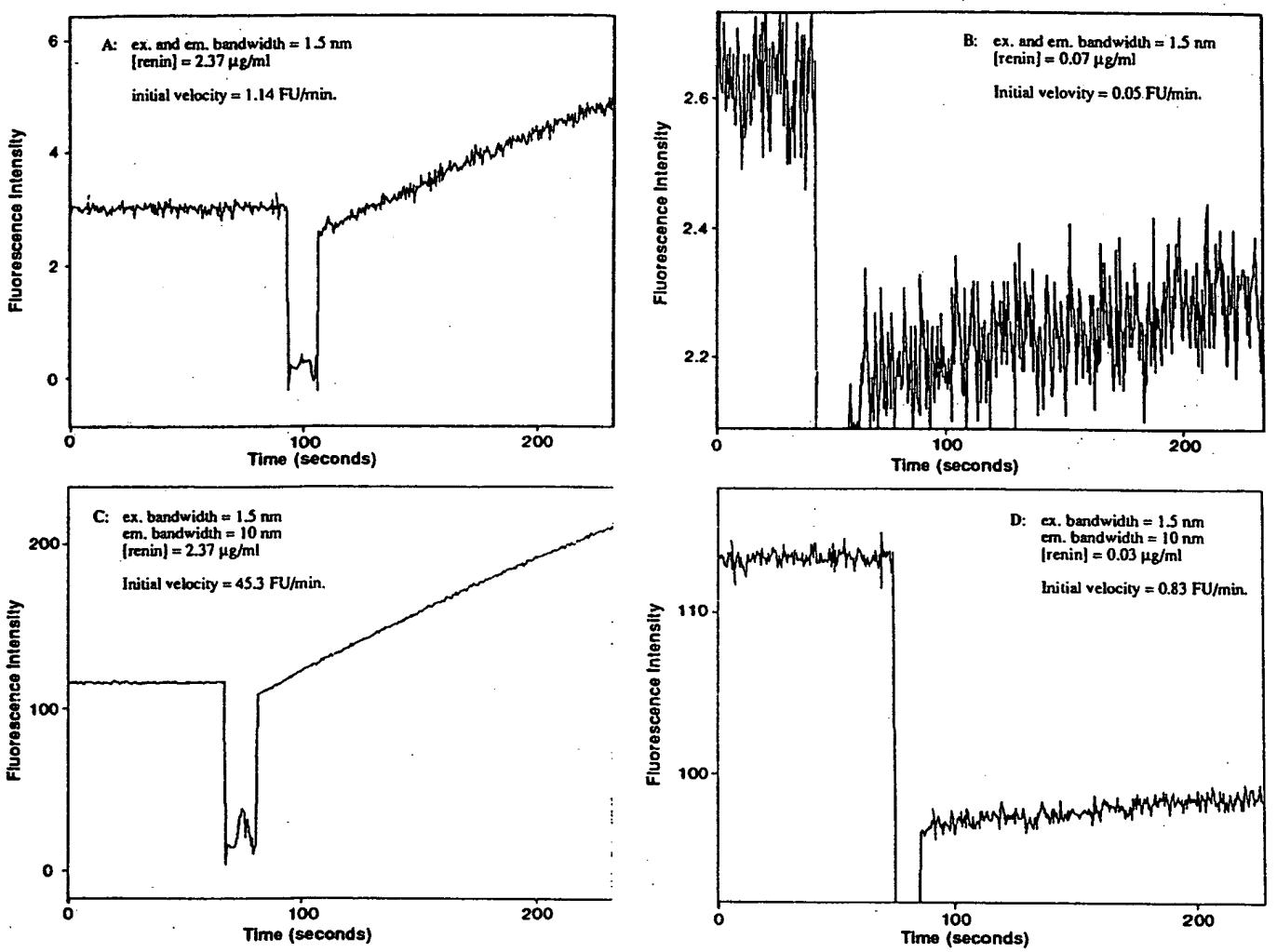

FIG. 2. Continuous fluorometric renin assay. Assays were performed on a Shimadzu RF-5000U spectrofluorometer following the protocol described under Materials and Methods. A 3 × 3-mm fluorescence cuvette containing 80 μl of a 0.05 M, pH 8.0, Tris buffer with 0.1 M NaCl and 1 mM EDTA and 10 μl of a 200 μM stock solution of fluorogenic substrate in DMSO was equilibrated at 37°C. Ten microliters of a renin stock solution (23.7 μg/ml), or an appropriate dilution of this sample, was added to initiate the reaction. Final substrate concentration is 20 μM in all assays, well above the K_m of 1.8 μM. The change in fluorescence was monitored for about 5 min. Initial rates of the reactions were then determined. The excitation and emission bandwidth and renin concentration for individual assays are as indicated. Assays shown in (B) and (D) represents the renin detection limit with emission bandwidth being set at 1.5 and 10 nm, respectively.

to as the R factor, which is approximately 1.8 (22). The calibration curves with emission bandwidth of 1.5 and 10 nm were shown in Figs. 4A and 4B, respectively. The fluorescence intensities were measured in the pH 8.0 Tris buffer used for renin assay in the presence of 10% DMSO. The data demonstrated a perfect linear correlation between the fluorescence intensity and the EDANS concentration. The correlation equations exhibited nonzero intercepts due to the small background fluorescence of the buffer.

Based on the linear correlation observed, initial velocities in μM/min, V_c, can be calculated from initial velocities in FU/min, V_i, using one of two methods. First, the equation

$$V_c\ (\mu M/min) = V_i\ (FU/min) \times (C_{ref}/I_{ref}) \times R,$$

which was described previously (15), can be used, where V_c and V_i are the initial velocity in concentration units and fluorescence intensity units, respectively, and C_{ref} and I_{ref} are the concentration and fluorescence intensity of a standard EDANS solution. R is the ratio of the fluorescence intensity of an equimolar solution of free EDANS to that of the cleavage product Val–Ile–His–Thr–EDANS.

Alternatively, the linear correlation equations shown in Figs. 4A and 4B can be used. In this case, the nonzero intercepts should be neglected since they cancel out when V_i (FU/min) is obtained [$V_i = (F_2 - F_1)/(t_2 - t_1)$].

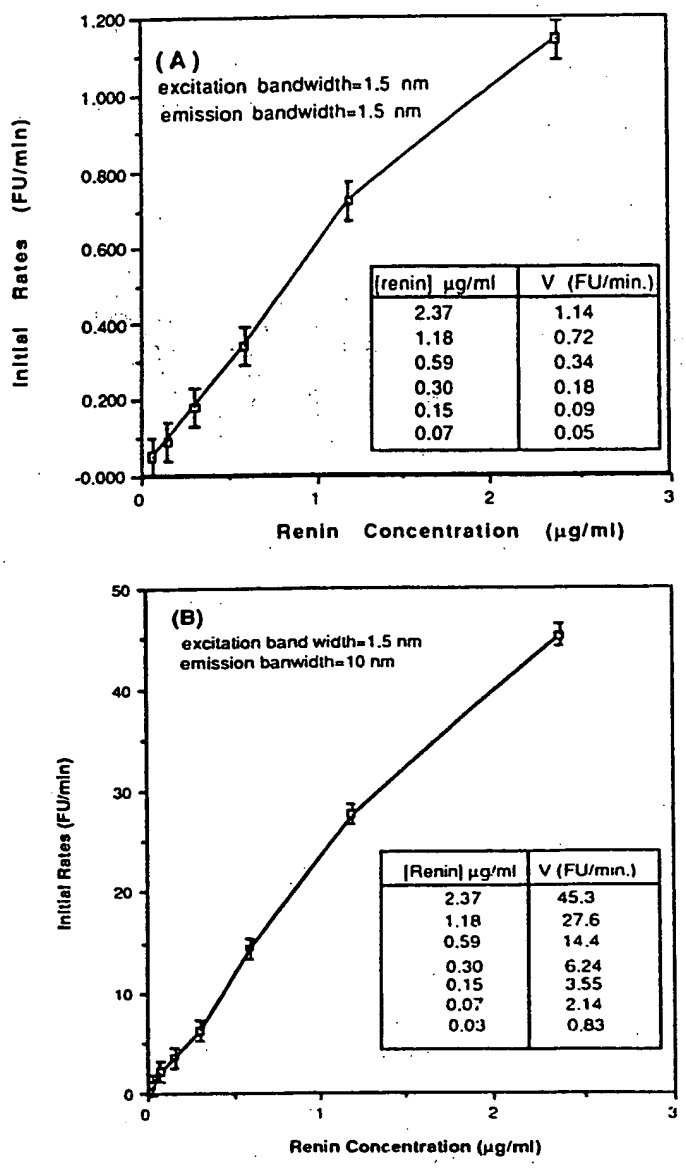

FIG. 3. Dependence of initial velocity (FU/min) on renin concentration. Initial velocities were determined using continuous assay format on a Shimadzu RF-5000U spectrofluorometer in a 0.05 M Tris buffer, pH 8.0, containing 0.1 M NaCl and 1 mM of EDTA at 37°C following the procedure described under Materials and Methods. A final substrate concentration of 20 μM was used in all the assays. The bandwidths are as indicated. Linear correlation ($R^2 > 0.98$) between the initial velocities and renin concentration was observed for both sets of data, excluding the [renin] = 2.37 μg/ml data point which showed significant deviation from linearity.

These two calibration methods are, in essence, identical and indeed gave the same results. Thus, the following equations were obtained for converting the initial velocities in fluorescence intensity units to concentration units: V_c (μM/min) = $0.51V_i$ (FU/min), when excitation and emission bandwidth is 1.5 nm, and V_c (μM/min) = $0.015V_i$ (FU/min), when excitation bandwidth is 1.5 nm and emission bandwidth is 10 nm (23).

With this calibration on hand, the sensitivity of our fluorogenic assay can then be assessed and compared

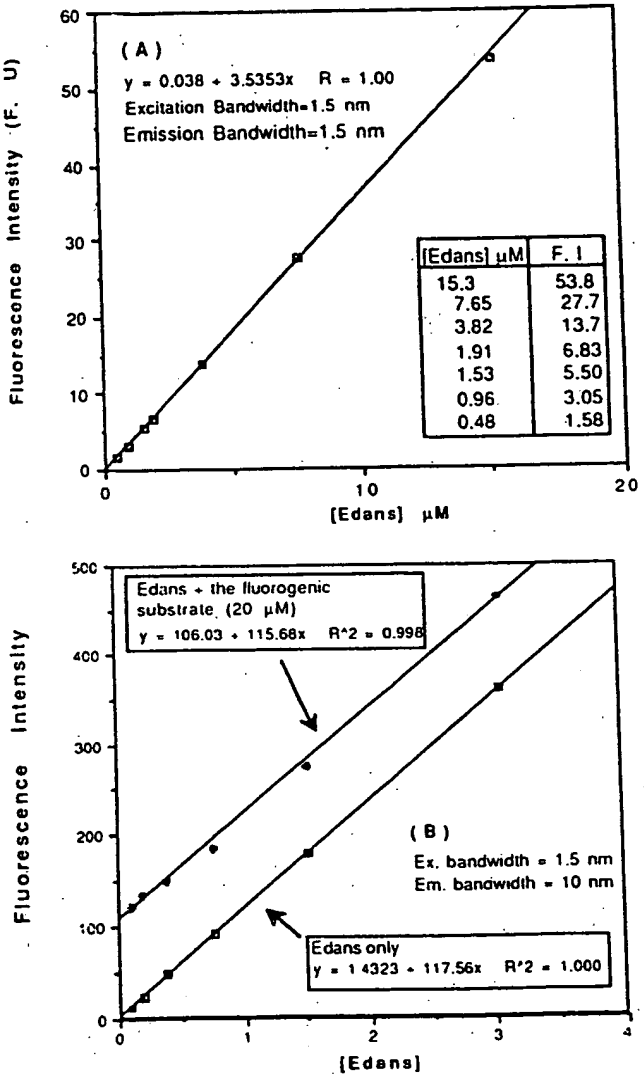

FIG. 4. Calibration of EDANS fluorescence on a Shimadzu RF-5000U spectrofluorometer. Fluorescence intensity of EDANS was measured in a 0.05 M Tris buffer, pH 8.0, using an excitation bandwidth of 1.5 nm. The emission bandwidth was 1.5 nm (A) and 10 nm (B), respectively. A 10-μl sample of an appropriate EDANS solution in DMSO was mixed with 90 μl of the buffer in a 3 × 3-mm fluorescence cuvette equilibrated to 37°C. The calibration curves (A and B, lower curve) showed perfect linear dependence of fluorescence intensity on concentration within the concentration range. The intercepts, which should be zero, are due to background fluorescence of the buffer. (B, upper curve) Concentration dependence of EDANS fluorescence in the presence of 20 μM of the fluorogenic substrate. Fluorescence was measured in a 3 × 3-mm cuvette using 80 μl of 0.05 M Tris buffer, pH 8.0, 10 μl of 200 μM stock solution of the fluorogenic substrate in DMSO, and 10 μl of an appropriate EDANS stock solution in DMSO.

with existing renin activity assays. The detection limits of literature renin activity assays were established inevitably based on data obtained with incubation time of 2–3 h (9,33). Based on the data shown in Fig. 2D (initial rate of 0.83 FU/min with 30 ng/ml of renin), a substrate hydrolysis rate of as low as 1.0 FU/h should be detectable if the incubation time is extended to 2–3 h. This would indicate a detection limit of 15 nM/h or 11 ng/ml/h in terms of production of the fluorescent peptide fragment Val–Ile–His–Thr–EDANS (M_r = 739). Extrapolation of these data based on the linear correlation between the initial rates and the renin concentration as shown in Fig. 3B gave a detection limit of 0.5 ng/ml of active renin. The indirect radioimmunoassay measuring angiotensin I production described by Haber *et al.* had a detection limit of about 1 ng/ml/h (9), whereas a direct radioimmunoassay using two antibodies against human renin showed a detection limit of 0.005 ng/ml (5 ng/liter) of active renin (33). Thus, it appears that our fluorogenic assay is about 10 times less sensitive than the indirect radioimmunoassay and 100 times less sensitive than the direct radioimmunoassay. This result is in line with the sensitivity of another renin assay reported recently using a different intramolecularly quenched fluorogenic substrate (34).

Depending upon the concentration of substrate used in a typical assay, it is possible that fluorescence measurements of the liberated Val–Ile–His–Thr–EDANS fragment may be affected by an inner filter effect, since these measurements are made in the presence of the quenching group DABCYL, either from the uncleaved substrate or the cleavage product DABCYL–gaba–Ile–His–Pro–Phe–Leu. This inner filter effect can introduce error into the calibration. In order to determine the extent of this effect, the fluorescence intensity of EDANS at several concentrations was measured in the presence of 20 μM fluorogenic substrate These data are plotted in Fig. 4B (upper curve). The results show a linear correlation between the EDANS concentration and the fluorescence intensity with a slope that is essentially identical to the slope observed in the absence of the fluorogenic substrate (Fig. 4B, lower curve). This shows that the inner filter effect is negligible in the presence of low concentration of the substrate (20 μM) used in this study, although it does not rule out the presence of such an effect at higher substrate concentrations.

Using the calibration results obtained above, the initial rates shown in Fig. 3 were converted into the units μM/min and plotted in Fig. 5. The two curves represent the results with emission bandwidths of 1.5 and 10 nm, respectively. Theoretically, these two curves should superimpose since the initial enzyme velocity must be independent of instrumental parameters, and this is the case, as shown in Fig. 5, within the limits of experimental error.

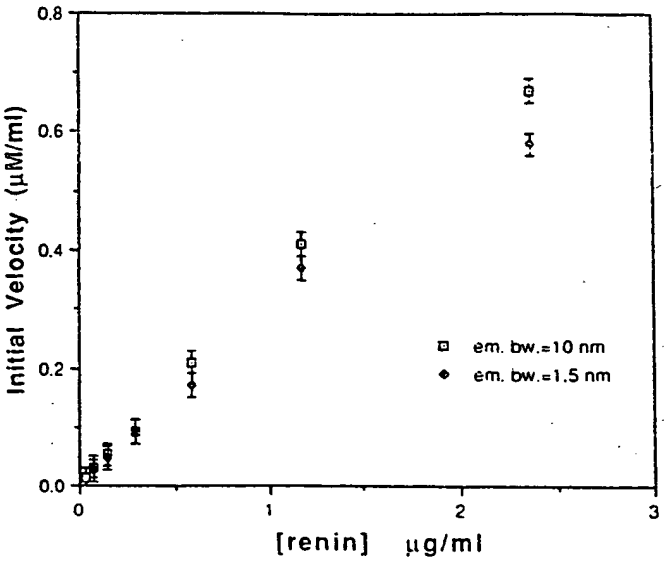

FIG. 5. Dependence of initial velocity (μM/min) on renin concentration. The initial velocity (FU/min) data in Figs. 3A and 3B were converted to μM/min units as described in the text.

This fluorogenic assay has been adapted to a 96-well microtiter plate format, which offers a number of distinct advantages. Multiple reactions and controls can be run simultaneously and the fluorescence signal for each reaction can be measured at fixed time points. In this format, data acquisition is quite efficient, enabling screening of large numbers of inhibitors and examination of many conditions that impact enzyme kinetics. It is also possible to obtain accurate kinetic parameters from time point data for each reaction, in instances where the reaction rates are sufficiently slow that deviation from linearity does not occur over a span of 5–10 min.

To demonstrate the utility of our fluorogenic renin substrate in a fixed-time assay format using a plate reader system, we have studied the pH dependence of activity of renin obtained by trypsin activation of recombinant prorenin under various solution conditions. Figure 6 represents assay data for one such experiment. In this experiment, activity of recombinant renin in the presence of 23% DMSO was examined at eight different pHs, ranging from pH 4.6 to pH 9.8. To each well was added 134 μl of an appropriate buffer, 26 μl of DMSO, 20 μl of a 200 μM DMSO stock solution of the fluorogenic substrate, and 20 μl of a renin sample, giving a total volume of 200 μl. Final substrate concentration was 20 μM and DMSO concentration was 23% (v/v). Each curve in Fig. 6 represents the time course of fluorescence change in a particular well. The results, illustrated graphically as an activity (relative fluorescence unit) vs pH plot, are shown in the inset of Fig. 6. This result demonstrated that the fluorogenic assay can be

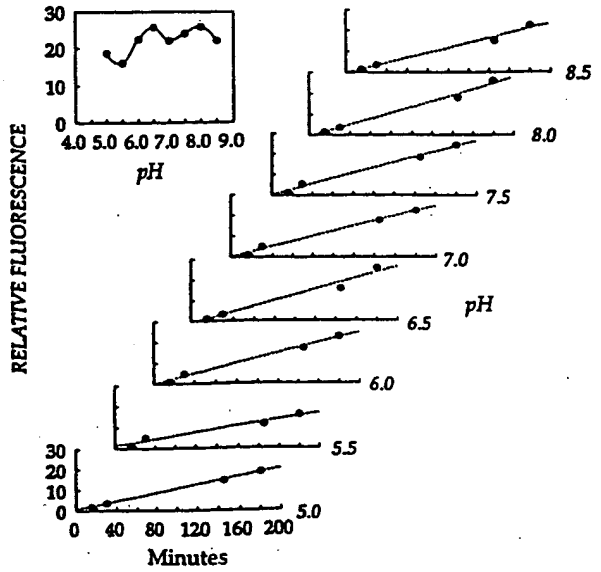

FIG. 6. pH activity profile of recombinant human renin in the presence of 23% DMSO. Fixed-time assays were performed on a Perkin–Elmer LS-50 luminescence spectrophotometer with a 96-well microtiter plate attachment. To each well was added 134 μl of an appropriate buffer, 26 μl of DMSO, 20 μl of a 200 μM DMSO stock solution of the fluorogenic substrate, and 20 μl of a renin sample, giving a total volume of 200 μl. Final substrate concentration was 20 μM and DMSO was 23 vol%. The assay buffer was a mixture of 150 mM each of Hepes, phosphate, CHES, and acetate. Buffer at each pH specified is prepared by titration of the buffer mixture to the desired pH with HCl or NaOH. The time course of fluorescence change in each individual well is represented by a curve. The fluorescence was read at fixed time points as shown using instrument parameters described under Material and Methods. The results were illustrated graphically as an activity (relative fluorescence unit) vs pH plot shown in the inset.

used in this format for high throughput kinetics analysis.

In summary, we have demonstrated in this paper that intramolecular fluorescence quenching due to resonance energy transfer can be applied successfully to fluorogenic substrates for aspartic proteases such as renin, which require relatively long peptide sequences for substrate recognition. Application of fluorescence quenching in protease substrates was first described about 2 decades ago and various examples utilizing this concept have appeared in the literature (24–32). Most of these substrates were short, poorly quenched, and were based on chromophores that were not generally applicable. Our previous work on HIV-1 protease substrates demonstrated that the DABCYL–EDANS pair has significant advantages over previous fluorophore–quencher pairs (15,17). The fluorescence quenching is extremely efficient due to the exceptionally good spectral overlap of EDANS emission and strong absorption band of DABCYL as well as the relatively long lifetime of EDANS. Thus, reasonable fluorescence quenching can be expected even if the EDANS and DABCYL are separated by a relatively long peptide. In addition, the EDANS and DABCYL moiety can be conveniently attached to the peptide termini, facilitating the synthesis of many different substrates. Application of this fluorogenic substrate technology to studies of a variety of protease drug targets will be described in future reports from our laboratory.

ACKNOWLEDGMENTS

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23. It should be emphasized that the calibration is certainly instrument dependent. Therefore, these results should not be applied to other instruments. The readers should perform their own calibration following the procedure outlined in this report.
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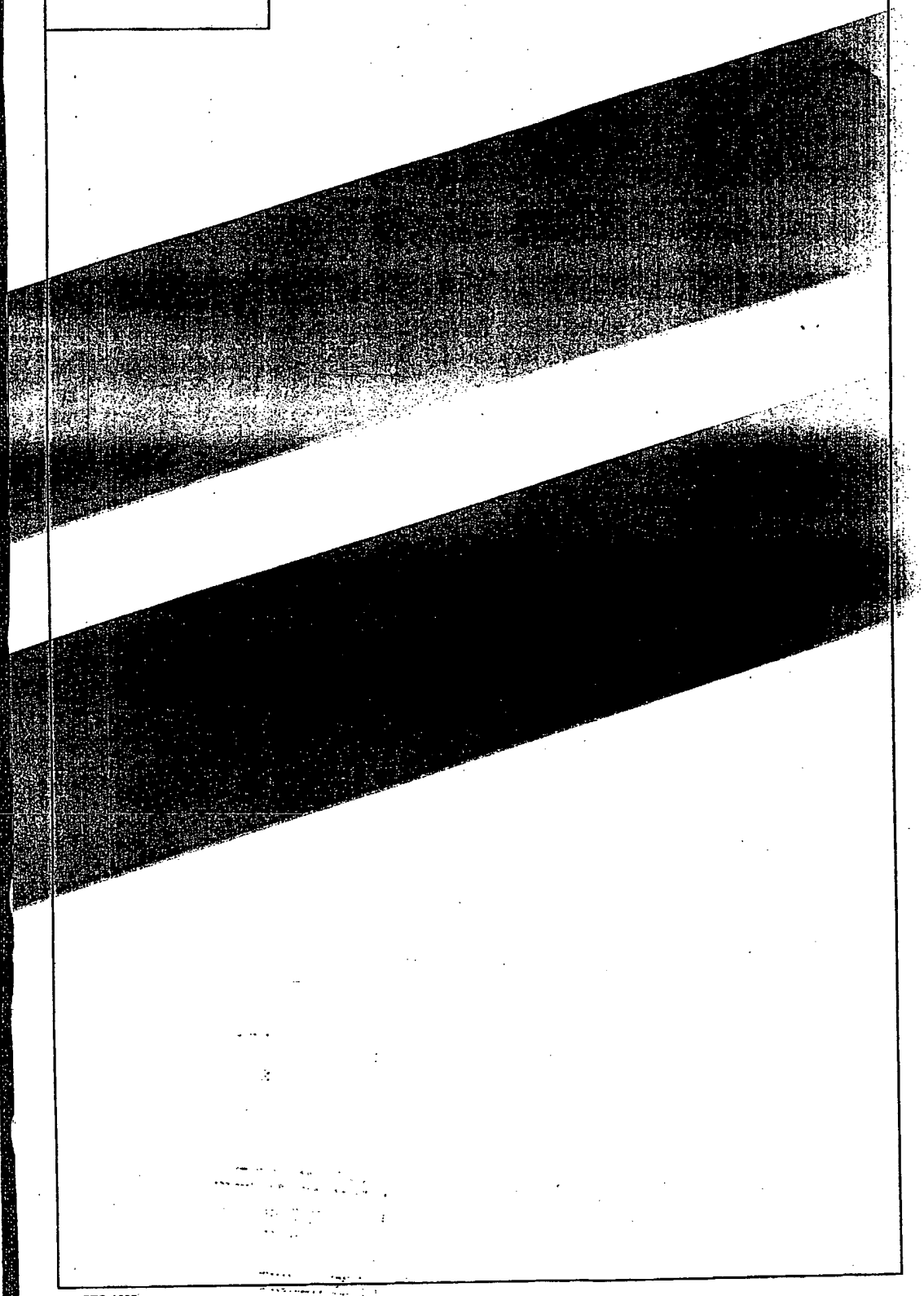

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EXHIBIT B

PATENT APPLICATION

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APPLICANTS
James J. Schmidt, Mt. Airy, MD ;
Robert G. Stafford, Ransom, WV ;

** CONTINUING DATA **********

** FOREIGN APPLICATIONS **********

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TITLE

Substrates for high-throughput assays of clostridial neurotoxin proteolytic activities

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Title: Substrates for High-Throughput Assays of Clostridial Neurotoxin Proteolytic Activities.

The invention consists of two types of compounds: (I), modified peptides or proteins that can serve as quenched-signal substrates in assays for the proteolytic activities of clostridial neurotoxins; and (II), modified peptides or proteins that can serve as immobilized substrates in assays for the proteolytic activities of clostridial neurotoxins. The assays are called "high-throughput" because lengthy processing steps such as centrifugation, solid-phase extraction, or chromatography are not required. Therefore, the assays can be readily adapted for use in automated or robotic systems. The term "clostridial neurotoxins" refers to the seven serotypes of neurotoxin (types A through G, inclusive) produced by *Clostridium botulinum* , and to tetanus toxin, produced by *Clostridium tetani.*

Type (I) compounds claimed:

Claim (1) is the following peptide:

N(alpha)-acetyl-S N R T R I D X A N Q R A Z R M L (amide)

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and "Z" is S-(fluoresceinyl)-cysteine. This peptide is a substrate for the proteolytic activity of type A botulinum neurotoxin.

Claim (2) is the following peptide:

N(alpha)-acetyl-L S E L D D R A D A L Q A X A S Q F E Z S A A K L K R -

K Y W W K N L K (amide)

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and "Z" is S-(fluoresceinyl)-cysteine. This peptide is a substrate for the proteolytic activity of type B botulinum neurotoxin.

Claim 3:

Any modified peptide or protein that can serve as a substrate for the proteolytic activity of any clostridial neurotoxin, said protein or peptide having been modified to contain a signal moeity on one side of the cleavage site, and a moeity on the other side of the cleavage site that quenches or diminishes the magnitude of that signal. When the substrate is cleaved, the two diffuse away from each other and the signal increases in proportion to the amount of cleavage that has occurred. Fluorescein and dinitrophenyllysine are examples of signal and quench moeities, respectively, but others are known.

Example of an assay using a type (I) compound:

In this case, the substrate was the peptide described in claim (1) above, and the enzyme was a recombinant preparation of type A botulinum toxin catalytic domain (i.e. the "light chain"). A solution of 30 micromolar peptide was prepared in water, buffered at pH 7.0 - 7.5. Before addition of enzyme, fluorescence was measured to obtain the background or "zero-time" fluorescence. Enzyme was then added to a concentration of approximately two micrograms per ml, and the resulting increase in fluorescence due to proteolysis of the peptide was measured vs. time. Assay temperature was 21° C. In the absence of enzyme, fluorescence changed very little with time, typically less than ± 5%. Results are shown in Fig. 1.

<u>Type (II) compounds claimed</u>:

Claim (4) is the following peptide:

N(alpha)-fluoresceinyl-G G G S N R T R I D E A N Q R A T R M L G G G-

C(amide)

This peptide is a substrate for the proteolytic activity of type A botulinum neurotoxin.

Claim (5) is the following peptide:

N(alpha)-fluoresceinyl-G G G L S E L D D R A D A L Q A G A S Q F E S-

S A A K L K R K Y W W K N L K G G C(amide)

This peptide is a substrate for the proteolytic activity of type B botulinum neurotoxin.

Claim (6):

Any modified peptide or protein that can serve as a substrate for the proteolytic activity of any clostridial neurotoxin, said protein or peptide having been modified so that it can be attached on one side of the proteolytic cleavage site to a solid or insoluble material. The attachment point can be on either side (i.e. C-terminal or N-terminal) of the cleavage site. On the other side of the cleavage site, the substrate contains a moeity that produces a measurable signal, such as (but not limited to) a fluorescent group or a radioactive isotope. When the proteolytic activity of a clostridial neurotoxin cleaves such a substrate, the product containing the signal is released into solution. Subsequently, the amount of signal in the soluble fraction is measured. Alternatively, the amount of residual bound signal can be measured following solublization with a protease such as trypsin.

Example of assays using type (II) compounds:

In this illustration, the substrates were the peptides described in claims (4) and (5) above, and the "solid material" to which the substrates were immobilized were 96-well microtiter plates that are chemically modified to contain maleimide groups. These plates are commercially available. A solution of 15 micromolar substrate was prepared in water, buffered at pH 8.0 - 8.5, and 100 microliters were added to each well. The sulfhydryl group of cysteine in the peptide reacted with a maleimide group on the surface of the well, forming a covalent bond, thereby anchoring one end of the peptide to the well. The wells were then washed to remove unreacted peptide, and then 100 microliters of enzyme were added to each well. In this example, type A botulinum neurotoxin was added to wells containing peptide (4), while type B was added to wells containing peptide (5). For both, the concentration of enzyme was approximately two micrograms per ml. The plates were incubated at 35° C. Aliquots were removed at various times and the fluorescence in each was measured. Results are shown in Fig. 2.

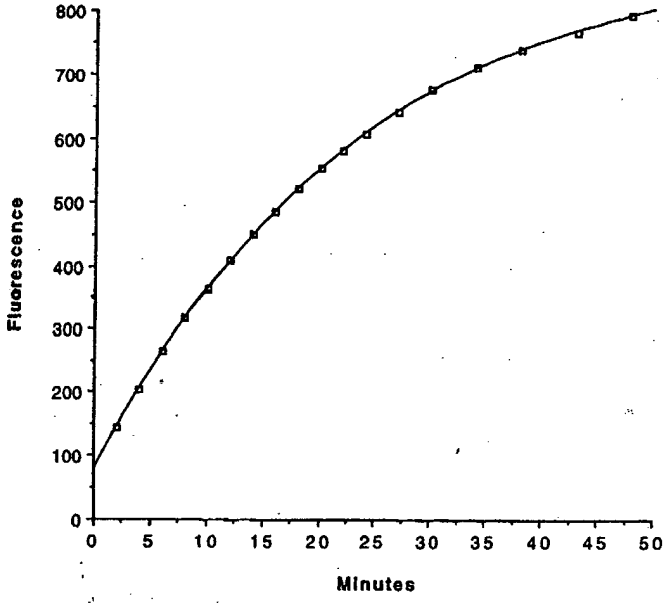

Fig. 1: Hydrolysis of peptide (1) by recombinant type A catalytic domain.

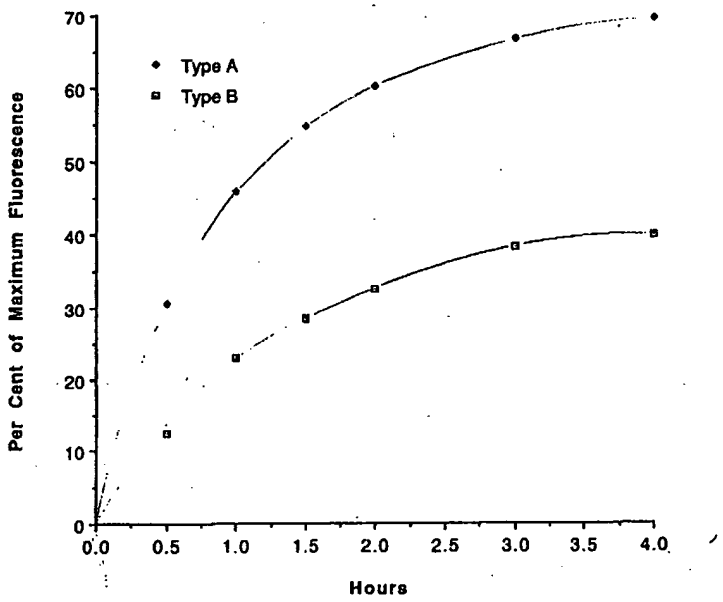

Fig. 2: Hydrolysis of immobilized substrates by types A and B botulinum neurotoxins.

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2).

Docket Number: 003/204/SAP RIID 00-38X Type a plus sign {+}

INVENTOR(S)/APPLICANT(S)

Last Name	First Name	Middle Initial	Residence (City and either State or Foreign country
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TITLE OF THE INVENTION (280 CHARACTERS MAX)

Substrates for High-Throughput Assays of Clostridial Neurotoxin Proteolytic Activities

CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS (Check all that apply)

[x] Specification	Number of Pages [3]	[] Small Entity Statement
[x] Drawing(s)	Number of Sheets [2]	[] Other (specify)

METHOD OF PAYMENT

[] A check or money order is enclosed to cover the Provisional filing fees
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Provisional Filing fee amount ($) 150.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.
[] No.
[x] Yes, the name of the U.S. Government agency and the Government contract number are: The United States Department of the Army.

Respectfully submitted,
Signature S.A. Pratt Reg No 39,441 Date Sept 25, 2000
Typed or printed name Elizabeth Arwine for Registration No. P45,867

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as Express Mail, Mailing label No. : EL688459841US in an envelope addressed to the
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By S.A. Pratt
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PROVISIONAL APPLICATION FILING ONLY

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In re Application of

Application Number	Filed
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Paper No. #2

I hereby request access under 37 CFR 1.14(a)(1)(iv) to the application file record of the above-identified ABANDONED application, which is identified in, or to which a benefit is claimed, in the following document (as shown in the attachment):

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Henry Duong
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